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Quantitative liquid chromatographic analysis of anthracyclines in biological fluids

The optimization, validation and application of a liquid chromatographic method with fluorescence detection for the simultaneous determination of the four most important anthracyclines (doxorubicin, epirubicin, daunorubicin and idarubicin) and their toxicologically relevant metabolites in plasma and saliva is described. The thesis also explored the possibilities of liquid chromatography coupled to fluorescence detection for trace analysis of anthracyclines in urine.

In **Chapter I** anthracyclines were introduced. They have the widest range of clinical use of any class of drugs in oncology. Current dosing, based on body surface area, leads to marked interindividual variations in efficacy and toxicity. Pharmacokinetic-pharmacodynamic relationships have been demonstrated by several research groups. The physicochemical properties, mechanism of action and pharmacokinetics were discussed. Anthracyclines have one toxicologically relevant metabolic pathway: the stereoselective reduction to 13-*S*-dihydro metabolites, which are generally denoted by the suffix "-ol". Acute and chronic adverse effects, mainly myelosuppression and cardiotoxicity, limit the dose and clinical activity. Since anthracyclines are highly toxic, occupational exposure has become a major concern.

Chapter II gave a literature overview of chromatographic methods for determination of anthracyclines in biological fluids. Anthracyclines are stable in most biological matrices, but not in blood. For clinical and pharmacokinetic studies, it is important to determine both the main compounds and their reduced metabolites. (Dis)advantages of deproteinization, liquid-liquid extraction and solid phase extraction as sample preparation techniques, were discussed. Since anthracyclines have natural fluorescent properties, fluorescence detection has been the detection method of choice for many years. Tandem mass spectrometric detection has been developed in recent years, and will probably become the detection technique of choice for the future, although there are some pitfalls. To assess occupational exposure, trace amounts in urine need to be quantified. A combination of a preconcentrating solid phase extraction step and liquid chromatography - tandem mass spectrometry can provide good sensitivity and selectivity.

Chapter III summarized the objectives of this thesis. The main goal was to develop an analytical method, that can be applied in routine laboratories to gain insight into individual pharmacokinetics of patients treated with anthracyclines.

In **Chapter IV** the synthesis of some commercially unavailable metabolites was described. An enzymatic synthesis formed stereoselectively the reduced metabolites, although reaction was never complete. Especially daunorubicin and idarubicin were better substrates than doxorubicin and epirubicin. HPLC coupled to fluorescence detection could monitor and predict the efficiency of the reaction. After reaction work-up, daunorubicinol and idarubicinol became available as methanolic

stock solutions. The aglycones doxorubicinone, daunorubicinone, idarubicinone, doxorubicinolone, daunorubicinolone and idarubicinolone were synthesized by acid hydrolysis of the corresponding glycosidic compounds. Although by themselves not being toxicologically relevant, their potential interference in the determination of clinically relevant anthracyclines and metabolites could be evaluated from then on.

The optimization of a liquid chromatographic separation for the simultaneous determination of the four most important anthracyclines and their respective reduced metabolites was the subject of **Chapter V**. Inclusion of epidaunorubicin, an anthracycline that is not administered to cancer patients, in the assay allows internal standardization of applications. Potential chromatographic interference of the above described aglycones was investigated. First the retention window was optimized. A gradient run on a 150x4.6 mm Purospher Star C18 column with a mixture of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with starting point at 24% solvent B and increment 1% solvent B/min created a suitable retention window. Then, resolution was enhanced by evaluating the column temperature: optimal column temperature was reached at 30 °C. Finally, combination of the retention and resolution data led to a two-step gradient: the initial gradient conditions, at a flow rate of 1 mL/min, were set at 24% solvent B, linearly increased to 30% solvent B in 7 minutes and to 58% solvent B in another 7 minutes. Inclusion of a flush and re-equilibration step yielded a total cycle time of 26 min. After analysis of the fluorescence spectra, an excitation and emission wavelength of 480 and 555 nm, respectively, provided optimal detection of the analytes.

In **Chapter VI** the extraction of anthracyclines from biological fluids was studied. The first part of the chapter discussed the extraction of plasma and saliva samples. Hereto a progressive optimization matrix was developed. In a first step, aqueous samples adjusted to 5 different pH-values (pH 3.0, 5.0, 7.0, 9.0 and 11.0) were extracted with a variety of organic solvents. The halocarbons dichloromethane and chloroform displayed the highest potential, especially when the pH of the aqueous layer prior to extraction was set to 7.0 and 9.0. Since the most polar anthracyclines had the lowest extraction yields, mixtures of a polar organic solvent and the halocarbons were tested in a second step. At the same time, the pH was fine-tuned by evaluation of phosphate buffers (at pH 7.0, 7.5, 8.0 and 8.5) and ammonium buffers (at pH 8.0, 8.5, 9.0 and 9.5). Ethanol was the polar organic solvent of choice. For phosphate buffers, an increase in pH and an increasing percentage of ethanol in the extraction solvent mixture improved extraction efficiencies. For ammonium buffers, a more complex pattern was seen. Instability phenomena could occur, and therefore ammonium buffers were abandoned. The combination of dichloromethane:ethanol (70:30, v/v) led to extraction efficiencies higher than 81% for all anthracyclines. Therefore, this optimized setting was applied plasma samples. However, an emulsion was obtained. This was solved by a two-step extraction: first a protein precipitation with ethanol, followed by a transfer of the supernatant after centrifugation, addition of dichloromethane and pH adjustment to 8.5. This procedure required an aliquot of 400 µL plasma. The same procedure was applied to saliva samples, but the starting volume was only 200 µL.

The second part of the chapter focused on the extraction for trace-analysis in urine. To avoid too large volumes of organic solvent, a preconcentrating solid phase extraction was performed.

Afterwards, a liquid-liquid extraction of the elution solvent under comparable conditions as optimized above enabled a further purification and enrichment of the sample. A SPEC phenyl column proved to be a promising SPE sorbent. A mixture of a 50-mM phosphate buffer pH 8.5 and ethanol (35:65, v/v) provided the best elution conditions. A wash solvent consisting of a 50-mM phosphate buffer pH 8.5 and ethanol (75:25, v/v) offered the best compromise between extraction efficiency and co-extraction of endogenous compounds. In spite of this extensive sample clean-up, trace amounts of anthracyclines in urine could not be reliably detected nor quantified by liquid chromatography coupled to fluorescence detection. A more selective detector, e.g. a triple quadrupole mass spectrometer, could solve this problem.

Chapter VII highlighted the importance of additives to stabilize chloroform, when chloroform is applied for extraction of anthracyclines. Chloroform exists in three varieties: without stabilizer, stabilized with an alcohol like ethanol, and stabilized with an unsaturated hydrocarbon. Without stabilization, chloroform degrades to form small amounts of free radicals, hydrochloric acid and phosgene. Stabilization with an unsaturated hydrocarbon is not always effective. Once phosgene is present, this leads to artifacts with amine containing compounds such as anthracyclines. Addition of an excess of ammonia during extraction can not overcome artifact formation. Addition of an alcohol in contaminated chloroform removes phosgene, but can not prevent artifact formation. Therefore, if chloroform is applied for the extraction of anthracyclines, ethanol stabilized varieties should be preferred.

In **Chapter VIII** the developed method for the simultaneous determination of the four most important anthracyclines and their respective reduced metabolites in plasma and saliva was validated according to selectivity, sensitivity, linearity, precision, accuracy, recovery and stability criteria. A very reproducible separation was developed, with intra-assay and inter-assay standard deviations in retention time no more than 0.03 and 0.21 min, respectively. There was no interference from blank samples, nor from samples spiked with the internal standard epidaunorubicin. Aglycones and frequently administered co-medication did not interfere in the analysis. The limit of detection and lower limit of quantification were 0.3 or 0.75, and 1 or 2.5 ng/mL, respectively, both in plasma and saliva. Linearity by means of weighted ($1/x$) regression was obtained from the lower limit of quantification up to 1000 or 2500 ng/mL for the parent drugs and up to 400 or 1000 ng/mL for the metabolites. If necessary, concentrations up to 10,000 ng/mL of the parent drugs in plasma could be quantified by a dilution of 1/10. Intra-assay and inter-assay precision relative standard deviation values were all less than 14% at low, medium and high levels, and below 17% at the LLOQ. Accuracy ranged between 91 and 113% at low, medium and high concentrations and between 83 and 118% at the LLOQ. The two-step extraction provided a robust sample preparation, with absolute recoveries between 78 and 88% in plasma, and between 70 and 79% in saliva, respectively. No autosampler instability was recorded. Benchtop, freeze-thaw and long-term stability samples fulfilled acceptance criteria, which means that the ratio of means (stability vs. control sample) was between 85 and 115%.

The previously developed and validated analytical methodology was applied to samples from 3 patients receiving FEC-chemotherapy in **Chapter IX**. As expected, dosage based on body surface

area could lead to interindividual variations in plasma concentrations. For these patients, this could be linked to haematological toxicity. In the first period after infusion, salivary concentrations of epirubicin increased. From then on, there seemed not to be a clear trend. Salivary epirubicinol concentrations could not be detected shortly after infusion, and always remained very low. Based on this method, it was impossible to detect an equilibrium between plasma and saliva concentrations within the first hours after dosing. A direct predictive power of saliva concentrations towards mucositis could not be assessed, since none of the patients developed such side effects. It should be stressed that the statements above are preliminary: a larger number of patients are an absolute prerequisite for definitive conclusions.

From the analytical side, future research should focus on tandem mass spectrometry as detection mode. In combination with modern (U)HPLC equipment, major improvements in sensitivity, selectivity and analysis time should be feasible. From the clinical side, more patients are essential for general conclusions. At present, the most promising aspect is to find a time point in plasma concentrations (e.g. after 2 hours) that can discriminate toxicity. This time point could then be used to routinely monitor all patients for haematological toxicity, eventually opening the possibility of individual dose adaptation.